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ORIGINAL RESEARCH

Synergistic Effect of Ubiquitin-Specific Protease 14 and Poly(ADP-Ribose) Glycohydrolase Co-Inhibition in BRCA1-Mutant, Poly(ADP-Ribose) Polymerase Inhibitor-Resistant Triple-Negative Breast Cancer Cells

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Purpose: The clinical benefits of poly(ADP-ribose) polymerase (PARP) inhibitors are limited to triple-negative breast cancer (TNBC) with BRCA deficiency due to primary and acquired resistance. Thus, there is a pressing need to develop alternative treatment regimens to target BRCA-mutated TNBC tumors that are resistant to PARP inhibition. Similar to PARP, poly(ADP-ribose) glycohydrolase (PARG) plays a role in DNA replication and repair. However, there are conflicting reports on the vulnerability of BRCA1-deficient tumor cells to PARG inhibition. This study aims to investigate the synergistically lethal effect of the PARG inhibitor COH34 and the ubiquitin-specific protease (USP) 14 inhibitor IU1-248 and the underlying mechanisms in BRCA1-mutant, PARP inhibitor-resistant TNBC cells.

Methods: The cytotoxicity of PARG inhibition alone or in combination with USP14 inhibition in the BRCA-mutant, PARP inhibitor-resistant TNBC cell lines, HCC1937 and SUM149PT, was analyzed using cell viability and proliferation assays and flow cytometry. The molecular mechanisms underlying the synergistic effects of IU1-248 and COH34 were evaluated by immunofluorescence staining, DNA repair reporter assays and Western blot analysis.

Results: It was found that HCC1937 and SUM149PT cells exhibited moderate responsiveness to PARG inhibition alone. To the best of our knowledge, this research is the first to demonstrate that the combination of IU1-248 and COH34 produces synergistic effects against TNBC cells in the same setting. Mechanistically, the blockade of USP14 by IU1-248 was shown to increase DNA damage and promote error-prone non-homologous end joining (NHEJ), as evidenced by the accumulation of γH2AX and 53BP1 in the nucleus and the activation of a reporter assay. Additionally, it was demonstrated that the inhibition of NHEJ repair activity attenuates the synergistic effects of concomitant PARG and USP14 inhibition. IU1-248 promotes NHEJ repair through the downregulation of the expression of c-Myc.

Conclusion: USP14 inhibition may be a plausible strategy for expanding the utility of PARG inhibitors in TNBC in BRCA-mutant, PARP inhibitor-resistant settings.

Keywords: PARG, USP14, NHEJ, c-Myc, BRCA, triple-negative breast cancer

Introduction

Breast cancer is the most commonly diagnosed cancer among women.[1] Triple-negative breast cancer (TNBC) accounts for approximately 15% of all breast cancer cases.[2] Compared to other breast cancer subtypes, TNBC is typically more aggressive and has a poorer prognosis. Several mutations have been shown to be associated with an increased risk of developing TNBC, including mutations in BRCA genes.[3] BRCA1 and BRCA2 are involved in homologous recombination (HR) and maintain genomic integrity.[4,5] Of note, 10–20% of patients with TNBC carry BRCA1/2 gene mutations.[6]

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Therapeutic strategies involving the use of poly(ADP-ribose) polymerase (PARP)1/2 inhibitors for tumors with BRCA1/2 mutations have been extensively researched.[7,8] Nonetheless, the responses of BRCA-mutated TNBC to PARP inhibitors Talazoparib (431 participants; hazard ratio for disease progression or death, 0.54; 95% confidence interval, 0.41 to 0.71; P<0.001) and Olaparib (302 participants; hazard ratio for disease progression or death, 0.58; 95% confidence interval, 0.43 to 0.80; P<0.001) appear to be less effective than those of BRCA-mutated ovarian cancer and have not been found to be durable.[9,10] This highlights the urgent need for the exploration of potential targets and novel drug treatment regimens for advanced TNBC with DNA repair defects.

Poly(ADP-ribose) glycohydrolase (PARG) is a potent enzyme that primarily catalyzes the rapid degradation of PAR added by PARPs.[11] Similar to PARP1/2, PARG is involved in DNA replication and repair.[12,13] Thus, PARG depletion or inhibition may exacerbate replication deficiencies in cancer cells. In contrast to PARP, PARG inhibition has been largely ignored and questioned as a therapeutic strategy. Conflicting results have been reported regarding the synthetic lethal targeting of BRCA1-mutant tumor cells with PARG inhibition.[14–16] However, several studies have reported the possibility that PARG inhibitors (PARGi) in combination with radiotherapy or cytotoxic drugs may exhibit efficacy in cancer cells.[15,17–21]

As one of the main posttranslational modifications, ubiquitination controls central signaling pathways in breast cancer.[22,23] Increasing evidence suggests that ubiquitination is a major regulator of DNA damage repair.[24,25] Ubiquitination is constantly reversed by specific deubiquitinating enzymes, of which ubiquitin-specific proteases (USPs) constitute the largest subfamily.[26] USP14 is one of the three proteasome-associated deubiquitinating enzymes that reversibly associates with the proteasome.[27] Increased expression of USP14 has been observed in multiple types of tumors, including breast cancer, suggesting poor tumor prognosis and indicating that USP14 may be a potential target for antineoplastic therapy.[28,29] Notably, USP14 has been reported to target proteins that participate in the DNA damage response.[30–32] A recent study suggested that USP14 is a target for radio-sensitization that regulates both HR and non-homologous end joining (NHEJ) in non-small-cell lung cancer.[32]

This study aimed to identify an effective drug regimen for treating BRCA-mutant, PARP inhibitor-resistant TNBC cells. The effects of PARG inhibition on the survival and growth of BRCA1-mutant, PARP inhibitor-resistant TNBC cells were examined. In addition, the potential of a USP14 inhibitor (USP14i) as an adjuvant to enhance the therapeutic efficacy of a PARG inhibitor (PARGi) in the same setting and its underlying mechanisms were investigated.

Materials and Methods

Cell Culture and Drugs

The TNBC cell lines, HCC1937 and SUM149PT, were acquired from ATCC (VA, USA) and Asterand Bioscience (MI, USA), respectively. HCC1937 cells were cultured in RPMI-1640 culture medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The SUM149PT cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 5μg/mL insulin, and 1 μg/mL hydrocortisone. The U2OS osteosarcoma cells were acquired from ATCC and were cultured in DMEM medium with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were regularly tested for Mycoplasma contamination. COH34 and IU1-248 were purchased from MedChemExpress (Shanghai, China) and Selleck Chemicals (TX, USA).

Clonogenic Assay

The TNBC cells were cultured in 96-well dishes for 24h and then stimulated with COH34 or IU1-248 alone or in combination as indicated in the figure legends. Media containing the drugs were changed every alternate day. At the end of the drug treatments, the cells were fixed and stained with 5% crystal violet for 20 min at room temperature. After cell imaging, 20% acetic acid solution was added to the plates to dissolve the crystal violet, and the absorbance at 570nm was measured using a multi-functional microplate reader (Bio-Rad Laboratories, CA, USA).

Cell Viability and Drug Synergy Assay

The HCC1937 and SUM149PT cells were transfected with PARG or c-Myc si-RNA (as described subsequently) or treated with COH34 or IU1-248 alone or in combination for 3 d. Cell viability was then examined using the CCK-8 assay, according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China). The

combined effects were analyzed using the Calcusyn software V2.0 to determine the combination index (CI).[33] CI < 1 indicates synergism.

Flow Cytometry

TNBC cell apoptosis was evaluated using the FITC-Annexin V apoptosis assay following the manufacturer's instructions (Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, cultured TNBC cells were treated with COH34 or IU1-248 alone or in combination for 48 h. Then cells were collected and incubated with FITC-Annexin V and PI. Apoptosis was measured by flow cytometric analysis using an Agilent NovoCyte Advanteon flow cytometer (Agilent Technologies, CA, USA).

Western Blot Analysis

Western blot analysis was performed as previously described.[34] Total cell extracts were prepared using RIPA buffer (MilliporeSigma, MA, USA) containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics, Basel, Switzerland). The primary antibodies used were PARG (27808-1-AP, Proteintech Group, Wuhan, China), USP14 (14,517-1-AP, Proteintech Group), Ku70 (10,723-1-AP, Proteintech Group), BRCA1 (22,362-1-AP, Proteintech Group), c-Myc (5605, Cell Signaling Technology, MA, USA), and β-Actin (66009-1-Ig, Proteintech Group). The secondary antibodies used were IRDye® 800CW Donkey anti-Rabbit IgG Secondary Antibody (926–32213, LI-COR Biosciences, NE, USA) and IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody (926–68070, LI-COR Biosciences). Blots were visualized using an Odyssey scanner (LI-COR Biosciences).

Immunofluorescence Assay

Immunofluorescence assays were performed as described previously.[35] Following incubating with γH2AX (2577, Cell Signaling Technology) and 53BP1 (4937, Cell Signaling Technology) antibodies, fluorophore-conjugated secondary antibodies (4412, Cell Signaling Technology) and DAPI (MBD0015, SigmaAldrich, MO, USA) were used. Cells were imaged and analyzed using an upright fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Plasmids and siRNA

The lentiviral vectors used in the present study were pCW57.1 (Addgene plasmid 41393) and pCW57.1 CMYC (Addgene plasmid 164145). The siRNA oligonucleotides were acquired from GenePharma (Suzhou, China). Cells were transfected with the siRNAs or the plasmids using Lipofectamine 3000® (Invitrogen, CA, USA) according to the manufacturer's instructions. Transfection efficiency was verified by Western blotting. The siRNA sequences were as follows: PARG: 5′-GCACTCTGTCTGCCAAATATT-3′; USP14: 5′-AGTTCTTAAGGATGTTAAATT-3′; Ku70: 5′-GAAGAGAACCTTGAAG CAAGT-3′; BRCA1: 5′-AGATAGTTCTACCAGTAAATT-3′; c-Myc: 5′-GCTTGTACCTGCAGGATCT-3′; negative control (NC): 5'-UUCUCCGAACGUGUCACGUTT-3'.

HR and NHEJ Reporter Assay

The indicated cells were transfected with pDRGFP (Addgene plasmid 26475) and I-SceI expression vector pCBASceI (Addgene plasmid 26477) for HR assay or with pimEJ5GFP (Addgene plasmid 44026) and pCBASceI for NHEJ assay using Lipofectamine 3000®. Following transfection, the cells were treated with 1.25 μM COH34 and 2.5 μM IU1-248 as single-agents or in combination for 48 h. The percentage of GFP-positive cells was measured using flow cytometry.

Statistical Analysis

Student's *t* test or two-way ANOVA with Bonferroni's or Tukey's multiple comparisons test was performed using GraphPad Prism version 9.0 (GraphPad Software, MA, USA). A *P*-value of <0.05 was considered to indicate a statistically significant difference.

Results

BRCA1-Mutant, PARP Inhibitor-Resistant TNBC Cells are Moderately Sensitive to PARG Inhibition

Synthetic lethal targeting of BRCA1-mutant tumor cells with PARG inhibition remains controversial.[14–16] This study investigated the effects of PARG inhibition on BRCA1-mutant TNBC cells. HCC1937 and SUM149PT TNBC cell lines harboring BRCA1 silencing mutations and resistant to PARP inhibitors were selected.[34] Genetic inhibition of PARG using siRNA inhibited the survival of both cell lines, as evidenced by CCK-8 assays (Figure 1A and B). HCC1937 and SUM149PT TNBC cell lines were further treated with the PARG-specific inhibitor, COH34.[15] Treatment with COH34 suppressed cell survival (Figure 1C and D) and attenuated clonogenic proliferation (Figure 1E) in both TNBC cell lines in a concentration-dependent manner. Collectively, these data indicate that PARG inhibition may lead to cellular cytotoxicity to a certain extent in BRCA1-mutant, PARP inhibitor-resistant TNBC cells.

USP14i IU1-248 Potentiates the Cytotoxic Effect of PARGi COH34 Against BRCA1-Mutant, PARP Inhibitor-Resistant TNBC Cells

Increasing evidence suggests that USP14 functions as a tumor promoter in breast cancer and that pharmacological inhibition of USP14 may suppress tumor cell proliferation.[36] USP14 has been reported to participate in the regulation of the DNA damage response; however, whether it is an effective target that enhances sensitivity to

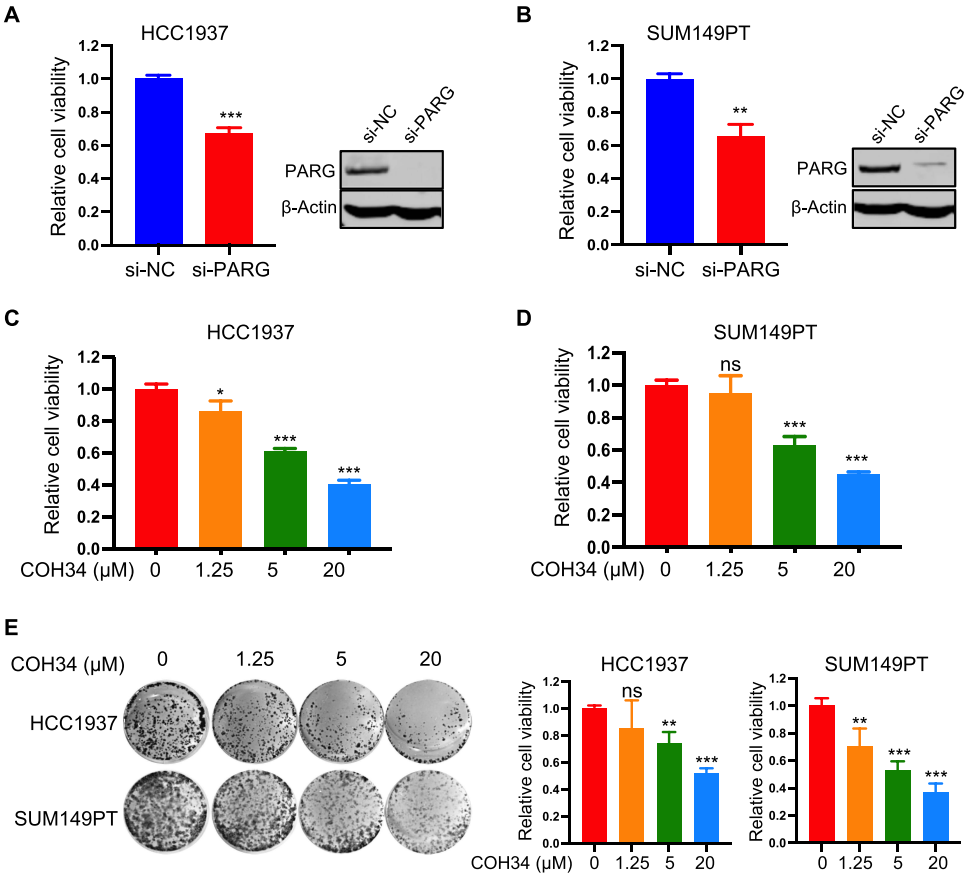

Figure 1 PARG inhibition results in survival and proliferation suppression of BRCA1-mutant, PARP inhibitor-resistant TNBC cells. (**A** and **B**) The relative cell survival was examined by CCK8 assay for HCC1937 cells (**A**) and SUM149PT cells (**B**) transfected with si-NC or si-PARG. PARG protein levels of Western blot analysis are shown. β-Actin was used as a loading control. (**C** and **D**) The relative cell survival was examined by CCK8 assay for HCC1937 (**C**) and SUM149PT (**D**) cells treated with COH34 at different concentrations. (**E**). Long-term relative cell viability was measured by crystal violet assay for HCC1937 and SUM149PT cells treated with COH34 at different concentrations.

Notes: The data are shown as the mean ±S.D. ns, not significant; **P*< 0.05; ***P*< 0.01; ****P*< 0.001 (Student's *t* test).

PARGi in TNBC remains unclear. The present study then aimed to investigate whether USP14 inhibition further enhances the cytotoxic effects of PARGi in BRCA1-mutant and PARP inhibitor-resistant TNBC cells. Compared to the vehicle, use of PARGi COH34 and USP14i IU1-248 alone moderately reduced cell viability. The combination treatment exerted more prominent cytotoxic effects at varying concentrations against BRCA1-mutant, PARPi-resistant HCC1937 and SUM149PT cells (Figure 2A). The synergistic effects of the combined treatments were determined using a clonogenic proliferation assay. Compared with observations of COH34 or IU1-248 treatment alone, the drug combination exerted more prominent inhibitory effects on cell proliferation (Figure 2B). Similarly, knockdown of USP14 with siRNA sensitized HCC1937 and SUM149PT cells to PARGi COH34, as analyzed by the cell viability assay (Figure 2C). Accordingly, the combination of COH34 and IU1-248 resulted in augmented apoptosis, as detected by the Annexin V/PI flow cytometric assay (Figure 2D). Collectively, these data suggest that USP14i enhances the cytotoxic effects of PARGi in BRCA1-mutant, PARP inhibitor-resistant TNBC cells.

USP14i IU1-248 Synergizes with PARGi COH34 to Induce DNA Damage and Promote NHEJ Repair

To elucidate the synergistic cytotoxic mechanism of IU1-248 and COH34 in BRCA1-mutant, PARP inhibitor-resistant TNBC cells, we evaluated whether USP14i IU1-248 exacerbated COH34-induced DNA damage using immunofluorescence analysis. While COH34 or IU1-248 alone moderately induced DNA damage, as indicated by accumulations of γH2AX nuclear foci (a molecular marker for DNA double-strand breaks [DSBs]), the drugs used in combination led to much more severe DSBs (Figure 3A). 53BP1 promotes NHEJ but antagonizes HR repair in BRCA1-deficient cells.[37,38] The present study noted a significantly increased number of 53BP1 foci in COH34/IU1-248-treated cells compared with those in cells treated with single agents or with vehicle (Figure 3B), indicating that USP14 inhibition may induce deficiency in HR repair and activate error-prone NHEJ repair. U2OS osteosarcoma cells are frequently used to study DNA damage responses, and U2OS reporter assay models have been established.[39] The present study directly analyzed changes in HR and NHEJ repair efficiencies in response to COH34/IU1-248 treatment in U2OS cells. The U2OS cells were transiently transfected with si-BRCA1 to induce BRCA1 defects. As determined using HR and NHEJ reporter assays, the combination treatment significantly increased the NHEJ activity in BRCA1-mutant U2OS cells, whereas COH34 or IU1-248 used alone moderately induced NHEJ activity (Figure 3C). IU1-248 alone or in combination with COH34 did not markedly affect HR activity (Figure 3D). These data suggest that PARGi and USP14i may be used synergistically to treat BRCA1-mutant tumor cells, promoting error-prone NHEJ repair.

NHEJ Repair Influences the Sensitization of BRCA1-Mutant, PARP Inhibitor-Resistant TNBC Cells to PARG-USP14 Co-Inhibition

To determine whether the synergistic response to PARG-USP14 co-inhibition was regulated by the enhanced activity of NHEJ repair, Ku70 (a central regulator of NHEJ signaling) was knocked down using siRNA (Figure 4A and B). HCC1937 and SUM149PT cells with silenced Ku70 expression exhibited a weakened response to the combination of PARGi and USP14i (Figure 4A and B). Ku70 knockdown abrogated this synergistic inhibitory effect on the viability of HCC1937 and SUM149PT cells (Figure 4C and D). Collectively, these data indicate that increased NHEJ activity may play a leading role in the sensitization of BRCA1-mutant and PARP inhibitor-resistant TNBC cells to PARG-USP14 inhibition.

USP14i IU1-248 Downregulates the Expression of c-Myc, Promoting NHEJ Repair and Sensitizing BRCA1-Mutant, PARP Inhibitor-Resistant TNBC Cells to PARGi COH34

A role for c-Myc in DNA damage and repair has also emerged.[40] A previous study reported that USP14 inhibition decreases c-Myc protein expression in esophageal squamous cell carcinoma cells.[41] This study evaluated whether c-Myc influences the synergistic inhibitory effects of IU1-248/COH34 on BRCA1-mutant and PARP inhibitor-resistant TNBC cells. Western blotting analysis revealed that IU1-248, used alone or in combination with COH34, significantly suppressed c-Myc expression in both HCC1937 and SUM149PT cells (Figure 5A). The knockdown of c-Myc using siRNA sensitized

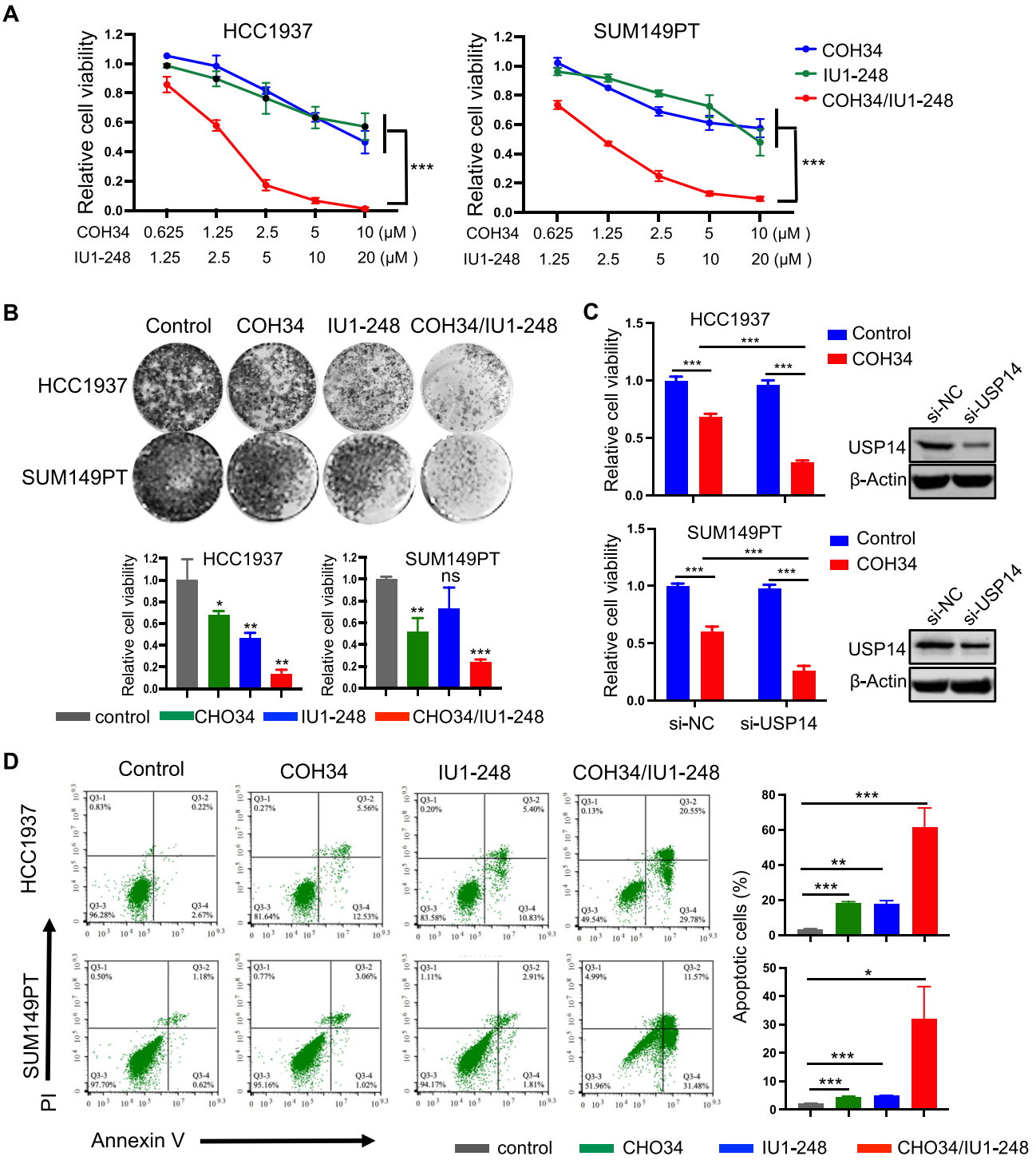

Figure 2 USP14i IU1-248 enhances the cytotoxic effect of PARGi COH34 on BRCA1-mutant, PARP inhibitor-resistant TNBC cells. (**A**). Dose-response curves of COH34 or IU1-248 alone or combined. The relative cell survival was examined by CCK8 assay for HCC1937 and SUM149PT cells treated with different concentrations of COH34 and IU1-248 for 72 h. The data are shown as the mean ± S.D. ***P< 0.001 (two-way ANOVA with Bonferroni's and Tukey's multiple comparisons test). (**B**). Long-term relative cell viability was measured by crystal violet assay for HCC1937 and SUM149PT cells treated with 2.5 μM COH34 and 5 μM IU1-248 as single-agents or in combination for 6 days. (**C**). Relative cell survival was examined by CCK8 assay for HCC1937 cells and SUM149PT cells transfected with si-NC or si-USP14. Cells were treated with or without 2.5 μM COH34 for 72 h. USP14 levels of Western blot analysis are shown. β-Actin was used as a loading control. (**D**). Flow cytometric apoptosis analysis of HCC1937 and SUM149PT cells following 2.5 μM COH34 and 5 μM IU1-248 as single-agents or in combination for 48 h. (**B-D**).

Notes: The data are shown as the mean ±S.D. *P< 0.05; **P< 0.01; ***P< 0.001 (Student's t test).

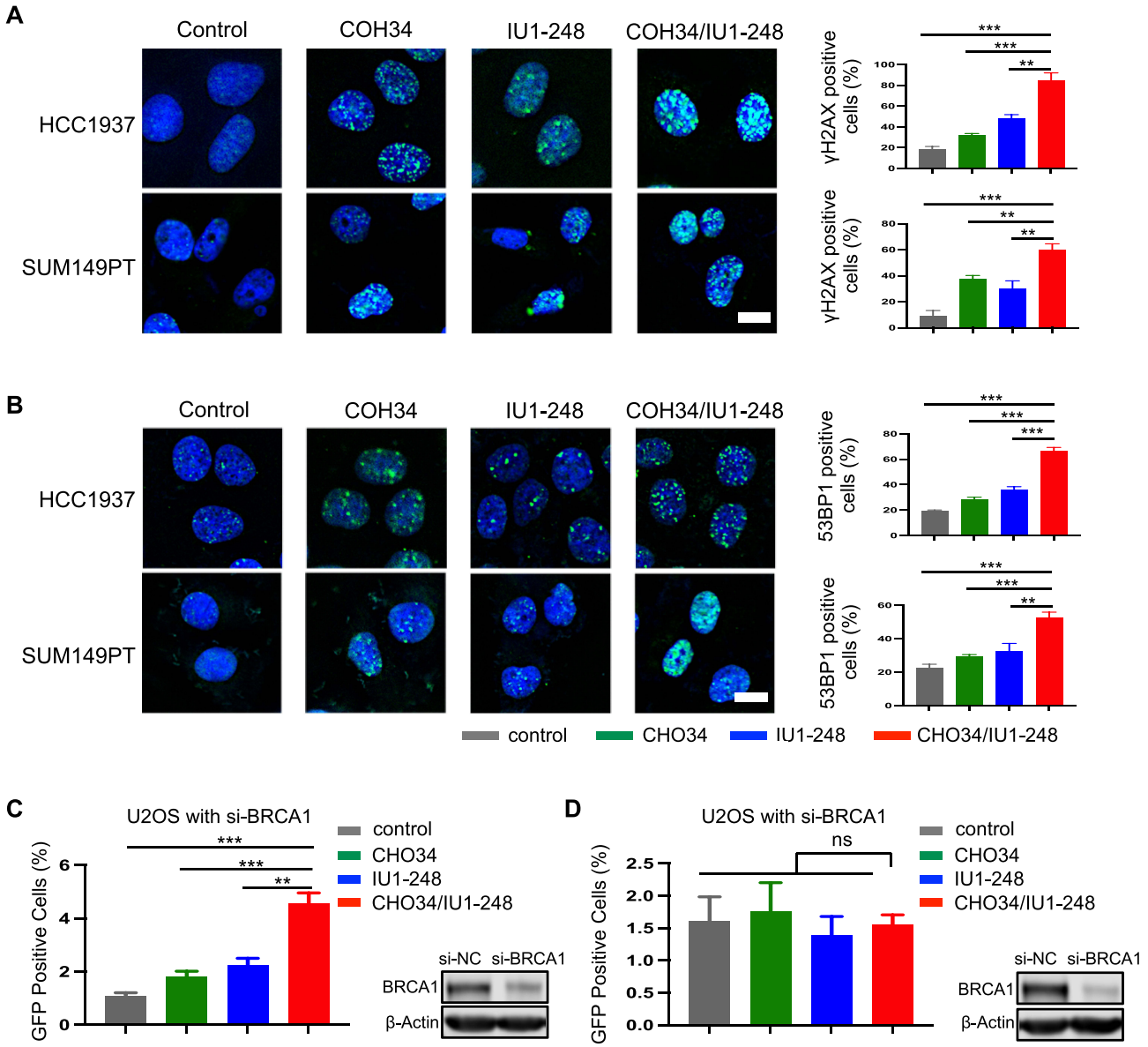

Figure 3 The combination of PARGi COH34 and USP14i IU1-248 induces DNA damage and promote NHEJ repairs. (**A** and **B**) Representative images of immunofluorescent staining for γH2AX (**A**) and 53BP1 (**B**) in HCC1937 and SUM149PT cells following 1.25 μM COH34 and 2.5 μM IU1-248 as single-agents or in combination for 48 h. Scale bar, 20 μm. Cells with more than five γH2AX or 53BP1 foci were identified as positive. (**C** and **D**) NHEJ (**C**) and HR (**D**) activities in U2OS cells following si-BRCA1 transfection and treatment of 1.25 μM COH34 and 2.5 μM IU1-248 as single-agents or in combination for 48 h were determined by the percentages of EGFP cells in the NHEJ and HR reporter assays. BRCA1 protein levels of Western blot analysis are shown. β-Actin was used as a loading control.
Notes: The data are shown as the mean ±S.D. ns, not significant; ***P*< 0.01; ****P*< 0.001 (Student's *t* test).

HCC1937 and SUM149PT cells to COH34, as determined using a cell viability assay (Figure 5B and C). Conversely, ectopic expression of c-Myc abrogated the synergistic cytotoxic effects of IU1-248/COH34 in HCC1937 cells (Figure 5D). Further molecular mechanistic analysis revealed that, compared with observations in the control cells, the HCC1937 cells with c-Myc ectopic expression exhibited an attenuated accumulation of γH2AX foci in response to IU1-248/COH34 combined treatment (Figure 5E). Accordingly, the BRCA1-interfered U2OS cells overexpressing c-Myc also showed downregulated NHEJ activity after the combined treatment (Figure 5F). Collectively, these results indicate that USP14i IU1-248 may promote DNA DSB and error-prone NHEJ repair through the downregulation of c-Myc, inducing synthetic lethality with PARGi COH34 (Figure 6).

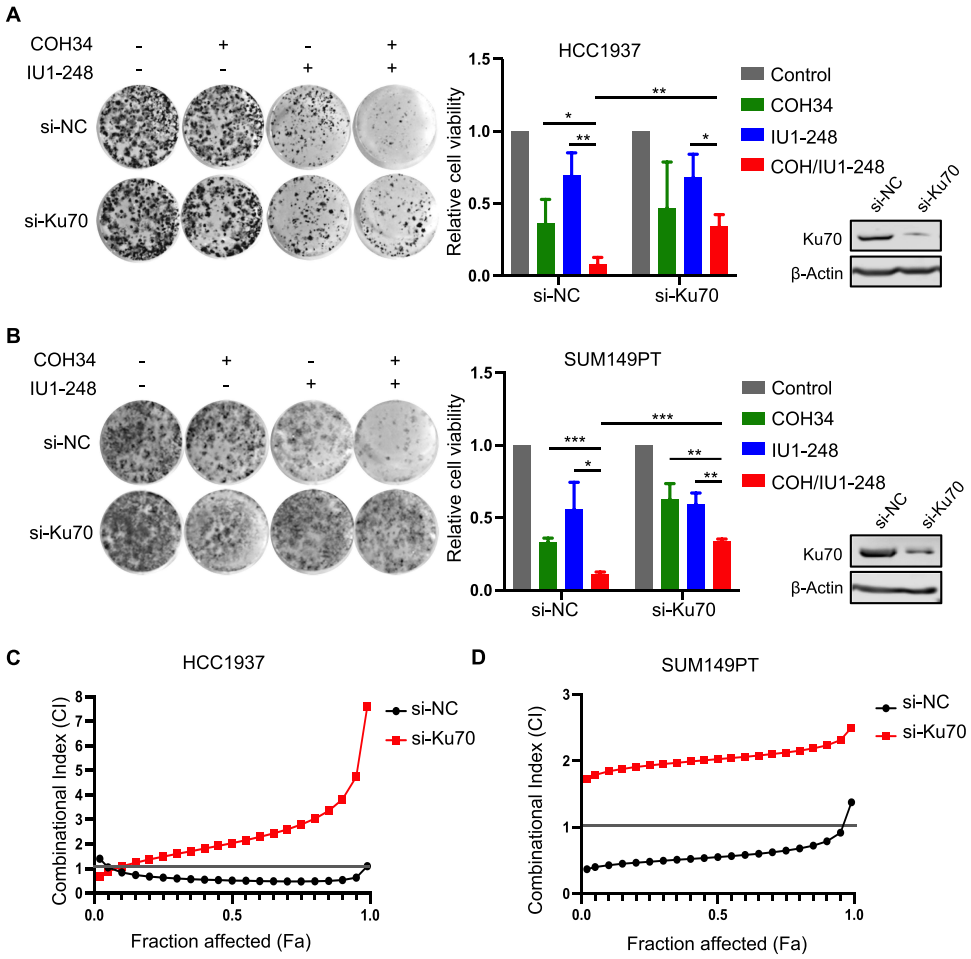

Figure 4 The NHEJ signaling is required for the combination effects of PARGi COH34 and USP14i IU1-248. (**A** and **B**) Long-term relative cell viability was measured by crystal violet assay for HCC1937 (**A**) and SUM149PT (**B**) cells. Cells were transfected with si-NC or si-Ku70 and treated with 2.5 μM COH34 and 5 μM IU1-248 as single-agents or in combination for 6 (SUM149PT) or 7 (HCC1937) days. Ku70 protein levels of Western blot analysis are shown. β-Actin was used as a loading control. (**C**) and (**D**) The synergistic effect of concomitant PARG and USP14 inhibition in HCC1937 (**C**) and SUM149PT (**D**) cells with or without Ku70-knockdown was measured by CCK8 assay after 72 h treatment.

Notes: The data are shown as the mean ±S.D. *P< 0.05; **P< 0.01; ***P< 0.001 (Student's t test).

Discussion

There is an urgent need to develop alternative active therapeutics for BRCA-mutant TNBC with primary or acquired resistance to PARP inhibition. Studies in the TCGA database have reported that a number of tumors, including breast cancer, exhibit higher PARG expression than that of normal tissues, suggesting the potential value of PARGi for further investigations.[18,21] Several novel PARGi have been explored for their favorable antineoplastic activities.[13,21] COH34 is a specific, cell-permeable, naphthalene-type PARGi that inhibits catalytically active PARG isoforms.[15] A recent study using COH34 revealed that PARG inhibition downregulates STAT3 activity and thus inhibits tumor cell growth and induces antitumor immune response.[42] Although studies have implicated PARG in DNA replication and repair,[12,13] the vulnerability of BRCA1-mutant tumor cells to PARGi remains controversial.[14–16] In the present study, using TNBC cell line models with BRCA1 silencing mutations and exhibiting resistance to PARP inhibitors, we found that genetic or pharmacological targeting of PARG exerts a suppressive effect on the survival and growth of BRCA1-mutant, PARP inhibitor-resistant tumor cells. IU1-248, an IU1 derivative, exhibits potent allosteric regulation of USP14 via steric blockade, that blocks the access of ubiquitin to the USP14 active site.[43] Notably, the present study demonstrates for the first time, to the best of our knowledge, that the combined use of COH34 and IU1-248 significantly inhibits cell survival and proliferation and promotes the apoptosis in BRCA1-mutant, PARP inhibitor-resistant HCC1937 and SUM149PT

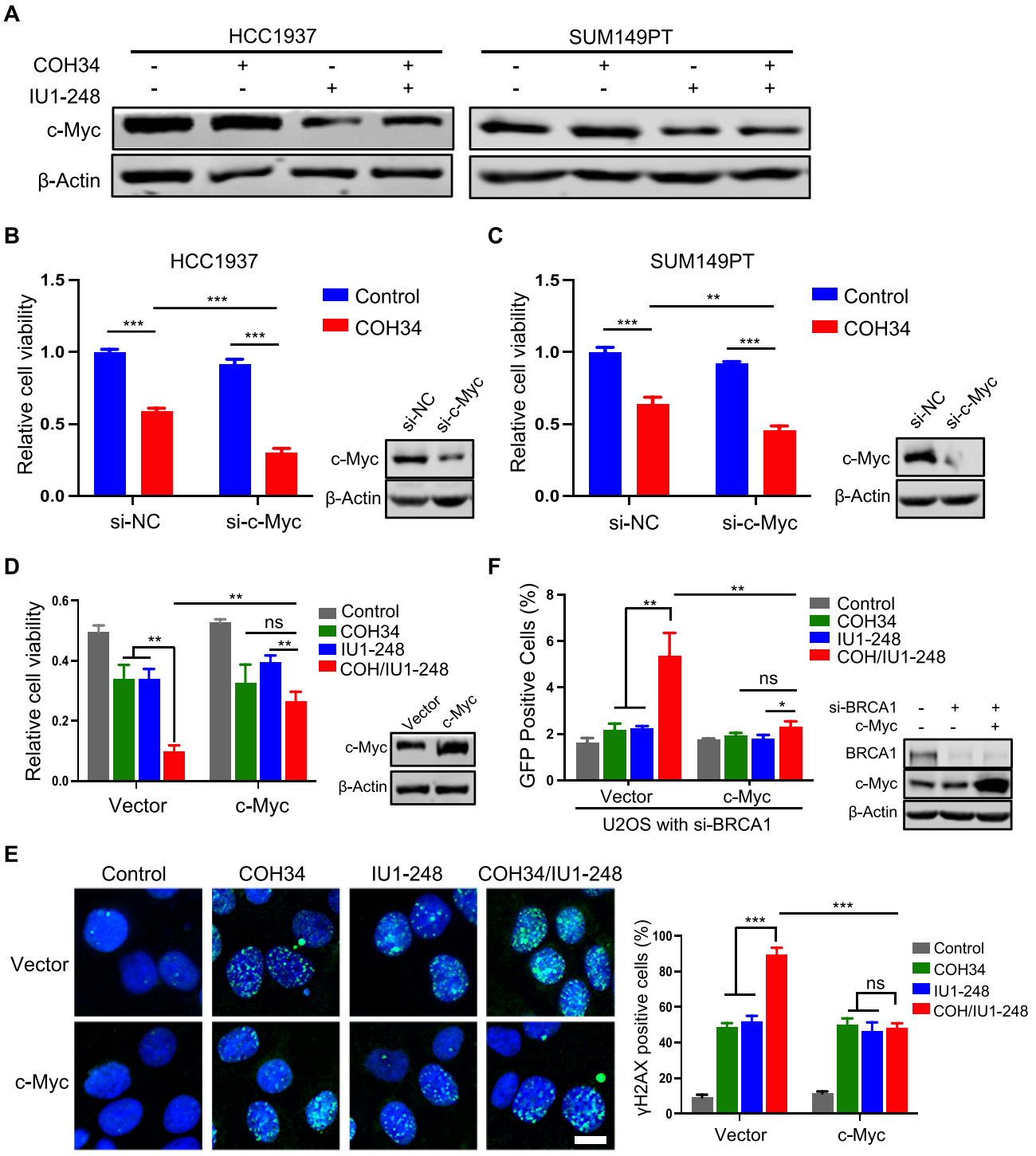

Figure 5 USP14i IU1-248 downregulates c-Myc, resulting in increased NHEJ repair and sensitizing BRCA1-mutant, PARP inhibitor-resistant TNBC cells to PARGi COH34. (**A**). Western blot analysis of c-Myc in HCC1937 and SUM149PT cells upon treatment with 2.5 μM COH34 and 5 μM IU1-248 as single-agents or in combination for 24 h. β-Actin was used as a loading control. (**B**) and (**C**) The relative cell survival was examined by CCK8 assay for HCC1937 cells (**B**) and SUM149PT cells (**C**) transfected with si-NC or si-c-Myc. Cells were treated with or without 2.5 μM COH34 for 72 h. c-Myc levels of Western blot analysis are shown. β-Actin was used as a loading control. (**D**). The relative cell survival of HCC1937 cells with or without c-Myc overexpression was measured by CCK8 assay after treatment with 2.5 μM COH34 and 5 μM IU1-248 as single-agents or in combination for 72 h. Western blot of c-Myc protein levels are shown. β-Actin was used as a loading control. (**E**). Representative images of immunofluorescent staining for γH2AX in HCC1937 cells with or without c-Myc overexpression. Cells were treated with 1.25 μM COH34 and 2.5 μM IU1-248 as single-agents or in combination for 48 h. Scale bar, 20 μm. Cells with more than five γH2AX foci were identified as positive. (**F**). NHEJ activity in U2OS cells with or without c-Myc overexpression following si-BRCA1 transfection and treatment of 1.25 μM COH34 and 2.5 μM IU1-248 as single-agents or in combination for 48 h were determined by the percentages of EGFP cells in the NHEJ reporter assay. BRCA1 and c-Myc protein levels of Western blot analysis are shown. β-Actin was used as a loading control.
Notes: The data are shown as the mean ±S.D. ns, not significant; **P*< 0.05; ***P*< 0.01; ****P*< 0.001 (Student's *t* test).

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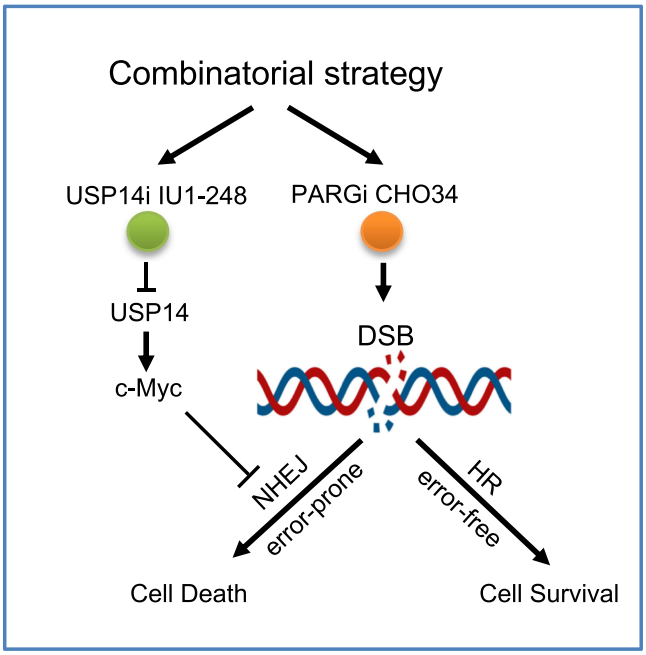

Figure 6 Schematic illustration to conclude the synergistic effect of USP14i and PARGi on BRCA1-mutant, PARP inhibitor-resistant TNBC cells. USP14 blockade increases the error-prone non-homologous end joining (NHEJ) through downregulation of c-Myc, and thus underlies PARG inhibitor sensitivity in BRCA-mutant, PARP inhibitor-resistant TNBC cells.

TNBC cells. Compared with observations when each treatment agent was used alone, the combined use of COH34 and IU1-248 had more notable therapeutic efficacy against HCC1937 and SUM149PT TNBC cells.

A balance between PARP-mediated PARylation and PARG-mediated dePARylation is vital for functional DNA DSBs. Similar to PARP, there is a potent rationale for targeting PARG in combination with DNA-damaging agents. The present study demonstrates that USP14 inhibition represents a therapeutic strategy to further sensitize BRCA1-mutant, PARP inhibitor-resistant TNBC cells to PARGi. NHEJ is the major repair pathway of DNA DSBs.[44] Recent studies have developed strategies of synthetic lethality with PARP inhibitors by facilitating error-prone NHEJ repair over HR repair.[44,45] In the present study, NHEJ plays a leading role in mediating the synergetic antineoplastic effect between PARGi and USP14i, as the inhibition of Ku70, a central regulator of NHEJ, efficiently blocks therapeutic synergy. Although other mechanisms may also result in a synergetic effect as separating the roles of PARG in DNA replication and DSB repair is not an easy task, the present study illustrates that regulating the choice of DSB repair may be explored to improve the therapeutic effect of PARGi against BRCA1-mutant and PARP inhibitor-resistant TNBC cells. Notably, a previous study indicated that targeting USP14 enhanced radio-sensitization in non-small cell lung cancer by regulating both NHEJ and HR repair.[32] In the present study, although increased 53BP1 (activating NHEJ and inhibiting HR of DNA DSBs) foci were observed in response to USP14i treatment in BRCA1-mutant HCC1937 and SUM149PT TNBC cells, it was found that neither USP14i alone nor in combination with PARGi altered the HR activity in BRCA1-depleted U2OS cells. These discrepant results suggest that the antineoplastic efficiency of USP14i and the underlying mechanisms may depend on genetic context. Future studies focusing on whether BRCA1 deficiency predicts sensitivity to PARGi and USP14i in combination and whether co-targeting PARG and USP14 is also effective in vivo may be necessary to extend the scope of the present study.

The role of c-Myc in DNA damage and repair remains controversial.[46–48] Studies have shown that c-Myc inhibits DNA damage repair and induces genetic instability.[49–51] Li et al provided evidence for the inhibitory effects of c-Myc on Ku70 DNA binding, DNA-dependent protein kinase catalytic subunit, and DNA end-joining activities.[49] In line with these findings, Data from the present study also indicate a potential negative regulatory role for c-Myc in NHEJ activity. However, several other studies have reported positive effects of c-Myc on Ku70 expression and DNA-dependent protein kinase catalytic subunit activity.[52,53] These controversial data suggest that the impact of c-Myc on DNA damage and

repair processes may be contingent on the context. The present study highlights the contribution of c-Myc-regulated NHEJ activity to the synergistic effects of concomitant PARG and USP14 inhibition in the BRCA1-mutant, PARP inhibitor-resistant TNBC cells.

Conclusion

The present study demonstrates that targeting PARG elicits DNA damage and reduces the survival and proliferation of BRCA1-mutant, PARP inhibitor-resistant TNBC cells. USP14 inhibition sensitizes BRCA1-mutant and PARP inhibitor-resistant TNBC cells to PARGi. Error-prone NHEJ plays a major role in mediating the synergy between PARGi and USP14i. USP14i IU1-248 promotes NHEJ repair through the downregulation of c-Myc, inducing synthetic lethality with PARGi COH34. Collectively, these data suggest that co-targeting PARG and USP14 may be a promising novel combination strategy for treating patients with BRCA1-mutant TNBC who are resistant to PARP inhibitors.

Abbreviations

TNBC, triple-negative breast cancer; HR, homologous recombination; NHEJ, non-homologous end joining; DSB, DNA double-strand break; PARP, poly (ADP-ribose) polymerase; PARG, Poly(ADP-ribose) glycohydrolase; PARGi, PARG inhibitor; USP, ubiquitin-specific protease; USP14i, USP14 inhibitor.

Data Sharing Statement

The data are available from the corresponding author on reasonable request.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests in this paper.

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